

Validation of the cantharidin-induced skin blister as an *in vivo* model of inflammation

Phong Huy Duc Dinh,^{1,3} Francis Corraza,² Kristel Mestdagh,¹
Zaina Kassenger,² Virginie Doyen¹ & Olivier Michel¹

¹Clinics of Immuno-allergy and of ²Hematology, CHU Brugmann (Université Libre de Bruxelles – ULB), Brussels, Belgium and ³Department of Immunology, Pham Ngoc Thach University of Medicine (PNTU), Ho Chi Minh City, Vietnam

WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Techniques to create aseptic inflammatory reactions provide information regarding acute inflammatory pathways and may be used to assess the anti-inflammatory properties of novel drugs.

WHAT THIS STUDY ADDS

- In this study, we have shown that the cantharidin-induced blister is a local inflammatory reaction, safe and well tolerated, with a good intra-subject inter-day reproducibility. This induced blister is inhibited by specific (anti-tumour necrosis factor (TNF)) and non specific (corticosteroids) systemic anti-inflammatory agents. This model could be of interest to evaluate anti-inflammatory agents in their early phase development.

Correspondence

Dr Olivier Michel MD PhD, CHU Brugmann/ULB – Université Libre de Bruxelles, place Van Gehuchten, 4, B – 1020 Brussels, Belgium.
Tel.: +32 2 477 2272
Fax: +32 2 477 2276
E-mail: omichel@ulb.ac.be

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AIM

Pharmacological profiling techniques, such as the cantharidin-induced skin blister, may be used to assess the anti-inflammatory properties of novel drugs. However, no data are available on the reproducibility of this technique or on the blocking effect of anti-inflammatory drugs, such as anti-TNF and corticosteroids.

METHODS

A group of 30 healthy subjects were randomized into three parallel groups treated with placebo, oral methylprednisolone 20 mg day⁻¹ for 7 days or anti-tumour necrosis factor (TNF) (adalimumab, Humira®, Abbott) 40 mg s.c. single dose. A first blister was induced at baseline and collected, immediately before the start of treatment and a second blister was obtained 7 days after the start of treatment. The total number of cells, the cell viability and the differential cell count were evaluated by two independent observers, who were blind to treatment. ANOVA was used to compare change from baseline among the three groups before pairwise comparisons.

RESULTS

Among the placebo group, there was no significant difference in the total cell count, neutrophils, eosinophils and monocytes between day 1 and day 7. Methylprednisolone inhibited the eosinophil influx in mean % (95% CI) (–1.0 (–1.7, –0.3); $P < 0.02$) and absolute ($P < 0.02$) values, while anti-TNF inhibited the neutrophil influx in mean % (95% CI) (–19.3 (–29.5, –9.1); $P < 0.01$) and absolute ($P < 0.05$) values.

CONCLUSIONS

The cantharidin-induced skin blister is a safe, well tolerated and reproducible procedure. Pre-treatment with anti-TNF or methylprednisolone inhibited the neutrophilic or eosinophilic trafficking, respectively. It could be useful in profiling anti-inflammatory drugs regarding their effects on the cellular inflammatory response.

Introduction

New drugs intended to treat inflammatory diseases are being developed to target the pathways of the inflammatory process. Controlled models of inflammation in humans are needed to screen for specific anti-inflammatory effects of new agents [1]. Three models of innate inflammation are based on the exposure of humans to small amounts of bacterial components, the intravenous or inhaled endotoxin exposure and the cantharidin-induced skin blister [2]. The skin blister model involves the topical application of the vesicant cantharidin to healthy skin, which causes *in vivo* leucocyte extravasation, cytokine release and clinical inflammation. Cantharidin is a defensive vesicant compound found within the haemolymph of blister beetles (*Meloidae coleoptera*). When applied to the skin it causes atraumatic acantholysis and blister formation. The technique has been standardized by Day *et al.* [3].

Techniques to create aseptic inflammatory reactions provide information regarding acute inflammatory pathways and may be used to assess the anti-inflammatory properties of novel drugs. For example, cantharidin-induced inflammation has been used to characterize the anti-inflammatory properties of low dose of aspirin [4] or to elucidate some local endogenous anti-inflammatory mechanisms [5].

To validate the cantharidin-induced skin blister method, testing is necessary with model anti-inflammatory agents. This was achieved by defining the inter- and intra-subject variability of the cantharidin-

induced skin blister, and evaluating the blocking effects of the reference anti-inflammatory drugs, anti-tumour necrosis factor (TNF) and corticosteroids [6].

Therefore, the aim of the present study was to evaluate the inter- and intra-subject variability of the skin blister reaction and the protective effects of anti-TNF and oral corticosteroid, compared with placebo, in a group of healthy subjects.

Methods

Subjects

Thirty healthy, male and female, non-smoker volunteers were included after written informed consent was obtained from each subject (mean age 31.0 (28–34) years; F/M: 16/14). Subjects were excluded if they used drugs within 2 weeks or over the counter medication.

The challenge is essentially as described by Day *et al.* [3]. Briefly, a filter paper disc is placed on the volar forearm skin of the subject, and then impregnated with 25 μ l freshly prepared 0.1% (w/v) cantharidin (Cantharone® R, Dormer Lab, Canada) in acetone (i.e. 25 mg per blister) (Figure 1). This dose of cantharidin is approximately 15% of the dose used in over the counter preparations for verruca treatment. The disc is allowed to air dry while maintaining contact with the skin, and a blister dressing is applied. The blister fluid is harvested 24 h after cantharidin application. The side of the blister is pierced with a sterile needle and the liquid is collected using a Gilson pipette (after having

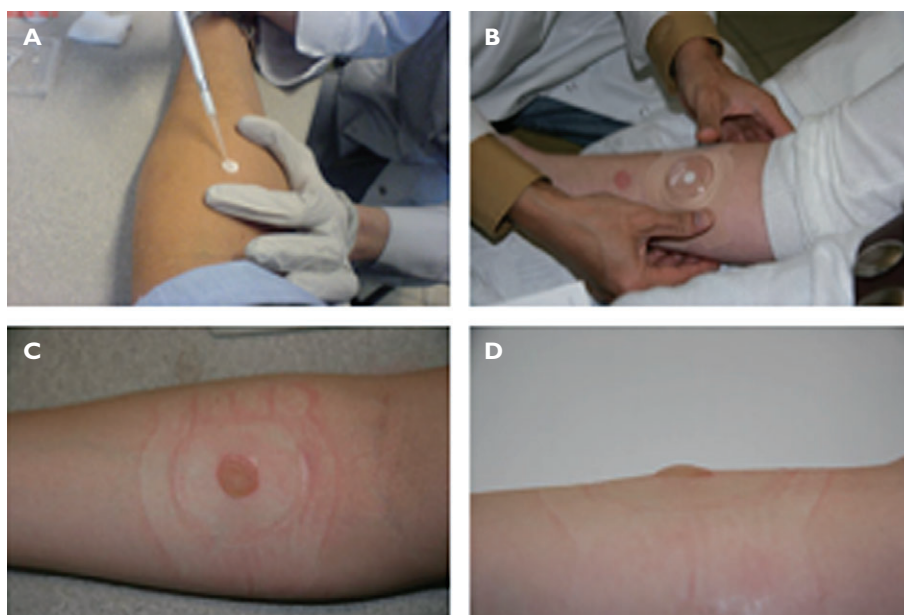


Figure 1

The procedure of the cantharidin-induced blister. (A) A filter paper disc is placed on the medium line of the forearm; 25 μ l of a 0.1% cantharidin solution in acetone is placed on to the filter paper disc; (B) Cover with an impermeable film and tape; then place a protective Pro-ophta eye dressing S cover; (C) By 24 h after induction there is a significant inflammation; (D) Blister shape

applied a very light pressure on the surface of the blister with a pipette tip). The volume (evaluated by weighing) is generally between 100 and 800 μL . The liquid is kept on ice at all times.

After filtration and centrifugation (400 g at 4°C for 10 min) the supernatant was frozen at -80°C while the pelleted cells were resuspended in BSA/PBS. The total number of cells was measured with a Thoma haemocytometer. The cell viability was assessed by the blue trypan method. A slide was prepared by centrifugation (Cytospin, Shandon Inc, Pittsburgh, PA, USA) and stained with May-Grünwald-Giemsa. The differential cell count was based on 400 cells by two independent observers (the results had to differ by less than 10%), who were blind to treatment.

Study design

The subjects were randomized into three open parallel groups treated with placebo, 20 mg oral methylprednisolone (Medrol®, Pfizer-Upjohn) once daily for 7 days or a single subcutaneous anti-TNF antibody, adalimumab (Humira®, Abbott) on day 1. The first blister was induced on day 0, around 08.00 h, the liquid being collected the following day (day 1) at the same time of the day. This blister was defined as a control blister. The placebo and methylprednisolone treatments were given on days 1 (immediately after sampling of the control blister) to 7; the anti-TNF was injected on day 1. A second blister was induced on study day 6 on the forearm (at least 5 cm distance from the first blister) the liquid being sampled on day 7 after recording the maximal diameter. A clinical follow-up visit was performed after 5 weeks (on day 42).

Good clinical practice

This study was conducted in compliance with Good Clinical Practice Guidelines of the International Conference on Harmonization. The study (EudraCT: 2008-005526-37) was prior approved by the Ethics Committee of the Institution (decision number CE2008/49) and the competent authorities in Belgium. Written informed consent was obtained in

each subject. The Clinical Research Unit of the Institution was responsible for study coordination.

Statistics

The results were expressed as mean or geometric mean \pm 95% confidence interval (CI). The absolute values of the cells were log transformed to normalize their distribution. Repeatability was assessed by plotting the differences between repeated measurements against the mean of the repeated measures, and testing whether the mean differences were significantly different from 0 (method of Bland & Altman) [7]. ANOVA was used to compare change from baseline among the three groups (placebo, methylprednisolone, anti-TNF). Pairwise comparisons between each active treatment and placebo, and between the two active treatments were performed. P values smaller than 0.05 were considered statistically significant.

Results

All but one subject developed an evaluable blister reaction. The procedure was well tolerated; three subjects complained of a sensation of painless pulling of the skin during the procedure, which disappeared completely less than 24 h after blister collection. There were no systemic symptoms in any subject. A slight pigmentation was still observed at the follow-up visit (i.e. 35 days after the procedure) in two subjects.

The characteristics of each group are summarized in Tables 1 and 2. Before treatment, the blister size was 16.5 (15.7, 17.3) mm, and the volume of recovered liquid was 298 (237, 360) μL . The geometric mean of the total viable cells was 977 (617, 1549) cells μL^{-1} , with a viability of 97.7 (95.6, 99.8) and a differential of 74.2 (68.4, 80.1) % polymorphonuclear neutrophils (PMN), 21.6 (16.3, 26.9) % monocytes, 2.9 (1.9–3.8) % lymphocytes and 1.1 (0.6–1.7) % polymorphonuclear eosinophils (PME). There was no significant difference between males and females.

Table 1

Demography and blister characteristics before (controls) and after treatment (placebo, methylprednisolone (PDN), anti-TNF

	Control	Placebo	PDN	anti-TNF
Subjects	30	10	10	10
Gender (F/M)	16/14*	4/6	5/5	7/3
Age (years, 95% CI)	31 (28, 34)	29 (23, 34)	33 (27, 40)	31 (25, 37)
Blister size (mm, 95% CI)				
Before	16.5 (15.7, 17.3)	17.3 (15.7, 18.8)	16.1 (14.5, 17.7)	16.1 (14.4, 17.7)
After	15.6 (14.9, 16.3)	15.2 (13.7, 16.8) [†]	15.6 (14.8, 16.4)	15.9 (14.3, 17.5)
Blister volume (μL , 95% CI)				
Before	298 (237, 360)	352 (236, 470)	248 (154, 342)	294 (156, 432)
After	211 (176, 245)	227 (152, 302)	202 (154, 250)	203 (127, 279)

*Age, blister size and volume were not significantly different among females compared with males.

[†] $P < 0.05$, paired t -test.

Table 2

Blister cell counts before and after treatments

Parameter†	Total	Placebo (1)	PDN (2)	P value‡ (1) vs. (2)	anti-TNF (3)	P value‡ (1) vs. (3)
Log viable cells (cells μl^{-1})						
Before	2.99 (2.79, 3.19)	2.96 (2.45, 3.47)	3.11 (2.82, 3.40)		2.90 (2.56, 3.24)	
After	2.96 (2.77, 3.14)	3.15 (2.88, 3.42)	3.00 (2.71, 3.29)		2.72 (2.27, 3.17)	
Change	-0.03 (-0.21, 0.15)	0.19 (-0.12, 0.50)	-0.11 (-0.45, 0.23)	0.16	-0.18 (-0.54, 0.19)	0.10
Log neutrophils (cells μl^{-1})						
Before	2.85 (2.64, 3.05)	2.78 (2.26, 3.31)	2.96 (2.65, 3.27)		2.79 (2.45, 3.14)	
After	2.76 (2.56, 2.97)	2.96 (2.67, 3.25)	2.85 (2.49, 3.21)		2.48 (2.04, 2.93)	
Change	-0.08 (-0.27, 0.10)	0.18 (-0.11, 0.46)	-0.11 (-0.48, 0.26)	0.19	-0.31 (-0.65, 0.03)	0.02
Log eosinophils (cells μl^{-1})						
Before	0.71 (0.44, 0.98)	0.28 (-0.11, 0.66)	0.95 (0.38, 1.52)		0.90 (0.45, 1.36)	
After	0.37 (0.11, 0.63)	0.43 (-0.08, 0.94)	0.16 (, 0.21, 0.53)*		0.52 (-0.08, 1.13)	
Change	-0.34 (-0.63, 0.04)	0.15 (-0.23, 0.53)	-0.79 (-1.28, 0.29)	0.004	-0.38 (-1.0, 0.24)	0.12
Log monocytes (cells μl^{-1})						
Before	2.24 (2.01, 2.46)	2.16 (1.57, 2.75)	2.48 (2.23, 2.73)		2.07 (1.69, 2.45)	
After	2.36 (2.18, 2.54)	2.53 (2.25, 2.82)	2.29 (2.09, 2.49)		2.25 (1.76, 2.74)	
Change	0.12 (-0.09, 0.34)	0.37 (-0.06, 0.80)	-0.19 (-0.49, 0.11)	0.03	0.18 (-0.24, 0.60)	0.48
Log lymphocytes (cells μl^{-1})						
Before	1.19 (0.93, 1.45)	1.03 (0.42, 1.64)	1.37 (0.96, 1.78)		1.17 (0.69, 1.64)	
After	1.11 (0.89, 1.33)	1.19 (0.66, 1.72)	1.27 (1.10, 1.45)		0.88 (0.43, 1.33)	
Change	-0.08 (-0.36, 0.21)	0.16 (-0.60, 0.91)	-0.10 (-0.53, 0.34)	0.52	-0.29 (-0.67, 0.09)	0.25
Neutrophils (%)						
Before	74.2 (68.4, 80.1)	72.7 (55.9, 89.4)	71.3 (65.4, 77.1)		78.8 (70.6, 87.1)	
After	66.8 (60.3, 73.3)	67.4 (55.1, 79.7)	73.5 (60.9, 86.1)		59.6 (48.0, 71.1)**	
Change	-7.4 (-14.3, 0.54)	-5.3 (-17.2, 6.7)	2.3 (-11.1, 15.6)	0.36	-19.3 (-29.5, 9.1)	0.05
Eosinophils (%)						
Before	1.1 (0.6, 1.7)	0.5 (0.0, 1.0)	1.5 (0.0, 3.0)		1.5 (0.6, 2.4)	
After	0.8 (0.2, 1.3)	0.7 (0.0, 1.7)	0.5 (0.0, 1.5)*		1.2 (0.0, 2.3)	
Change	-0.4 (-0.8, 0)	0.2 (-0.3, 0.7)	-1.0 (-1.7, 0.3)	0.004	-0.4 (-1.1, 0.3)	0.15
Monocytes (%)						
Before	21.6 (16.3, 26.9)	23.0 (7.8, 38.2)	24.5 (19.0, 30.0)		17.2 (10.0, 24.5)	
After	29.9 (23.7, 36.0)	28.8 (17.2, 40.3)	23.6 (12.2, 34.9)		37.3 (25.7, 48.8)**	
Change	8.3 (1.8, 14.7)	5.8 (-4.0, 15.5)	-1.0 (-13.1, 11.2)	0.35	20.0 (9.3, 30.8)	0.04
Lymphocytes (%)						
Before	2.9 (1.9, 3.8)	3.5 (1.0, 6.0)	2.7 (1.1, 4.3)		2.4 (1.2, 3.6)	
After	2.5 (1.5, 3.5)	3.2 (0.7, 5.6)	2.4 (0.9, 3.9)		1.9 (0, 3.8)	
Change	-0.4 (-1.5, 0.8)	-0.4 (-3.1, 2.4)	-0.3 (-2.2, 1.7)	0.95	-0.5 (-2.7, 1.7)	0.92

* $P < 0.05$; ** $P < 0.01$. Paired t -test to compare the blister cell counts before and after placebo, PDN and anti-TNF.

†Values are expressed as arithmetic mean (95% confidence interval). PDN, methylprednisolone.

‡Unpaired t -tests were used to compare the changes after placebo (1) with the changes after methylprednisolone (2) or anti-TNF (3).

The study population included 30 subjects who were randomized in a ratio 1:1:1 between three treatment groups: placebo, anti-TNF and oral corticosteroids. There was no significant difference between the groups regarding the size/volume or the cell counts of the control blisters before treatment. The intra-subject repeatability of the method was evaluated in the placebo group, by comparing the characteristics of the blisters on day 1 and day 7 (Figure 2). The Bland & Altman analysis showed that the measurements of number of total cells, PMN, PME, monocytes, weight and size were not statistically different between day 1 and day 7 and the variability of measurement was not high thus suggesting good reliability of the method (Figure 2).

Drug effects were then evaluated for total cells, PMN, PME and monocytes. The oral corticosteroid treatment significantly inhibited the influx of eosinophils in the blister fluid and absolute values while it did not have any signifi-

cant effect on total cells, monocytes or PMN (see Table 2 and Figure 3). The treatment with anti-TNF inhibited the influx of neutrophils in the blister fluid (both in % and absolute value) and of monocytes (in % but not in absolute value) while it did not have a significant effect on the total cells or on the absolute number of PME (see Table 2 and Figure 3). The diameter and/or volumes of the blisters were modified neither by oral corticosteroid, nor by anti-TNF (Table 1).

The changes of the cell counts after placebo were then compared (by repeated measures ANOVA) with the changes after treatment with corticosteroids or anti-TNF (Figure 4). The F -tests were significant for PMN % ($F^2_{27} = 4.32, P = 0.02$), PME % ($F^2_{27} = 4.63, P < 0.02$), mono % ($F^2_{27} = 4.90, P = 0.02$), log PME ($F^2_{27} = 4.87, P = 0.02$), and nearly significant for log PMN ($F^2_{27} = 2.71, P = 0.08$) and log monocytes ($F^2_{27} = 2.74, P = 0.08$). The change of PMN count (both in absolute and %) among the subjects treated with anti-TNF and the change

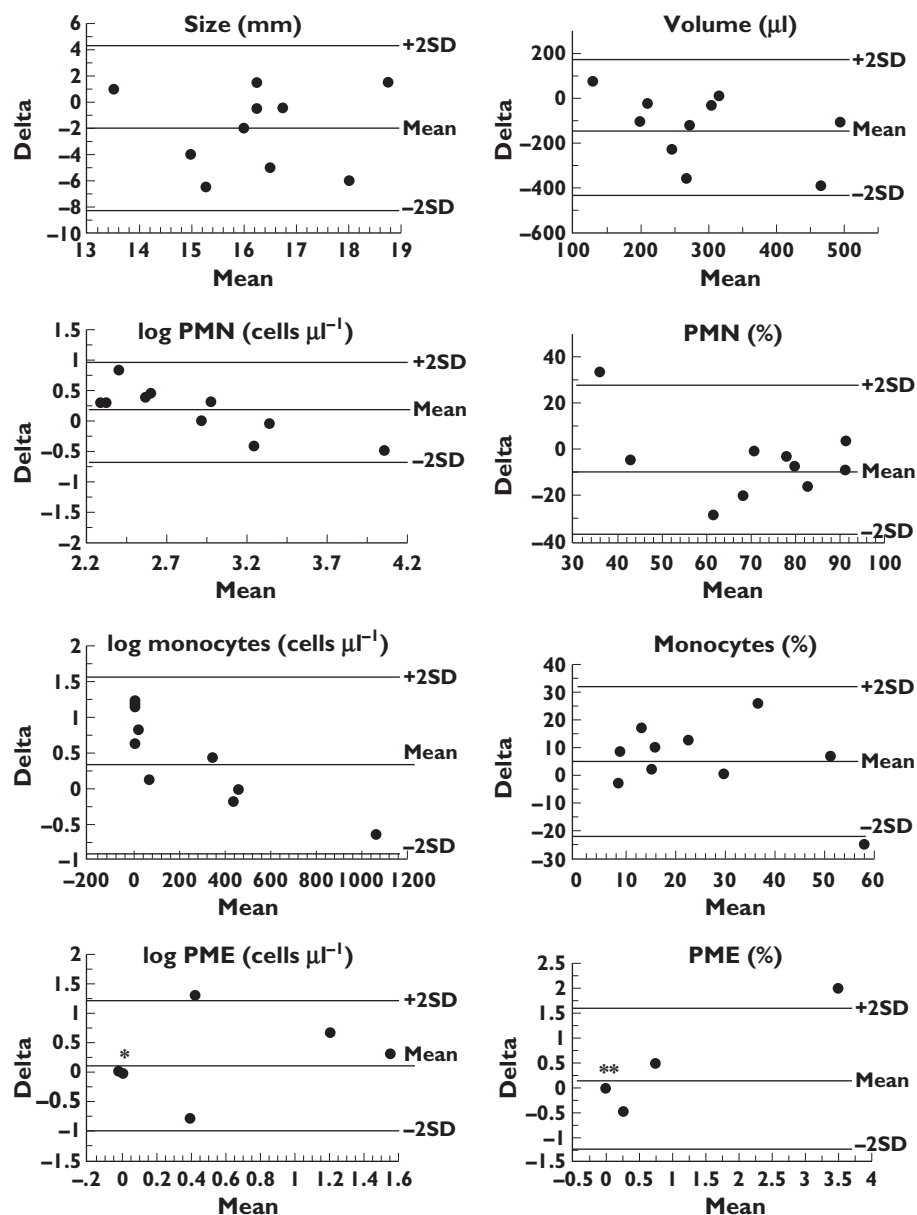


Figure 2

Assessment of repeatability of the cell counts of the blisters. The differences against the means, of the blister cells counts, between the day 1 and day 7 among the placebo group. Limits of agreement are the mean \pm 2 SD. * and ** includes five and seven subjects

of the PME count (both in absolute and %) were statistically attenuated compared with placebo.

The individual values of the changes in PMN and PME after a treatment with oral corticosteroids or subcutaneous anti-TNF are shown in Figure 5.

Discussion

The results of the present study show that a) the cantharidin-induced skin blister is a safe and well tolerated procedure, b) the inter-subject variation of the amplitude

of the clinical response is low, c) the intra-subject inter-day reproducibility of leucocyte accumulation in the blister fluid is significant, d) pre-treatment with methylprednisolone inhibits the eosinophilic inflammatory response and e) pre-treatment with the anti-TNF agent adalimumab inhibits neutrophil trafficking.

The skin-window chamber technique is based on a local injury to induce exudation and leucocyte migration. Originally, a physical trauma was applied either by abrasion [8], high-speed drill [9] or negative pressure [10]. In the 1960s, a chemical method was published, based on the use of cantharidin causing acantholysis and blister formation

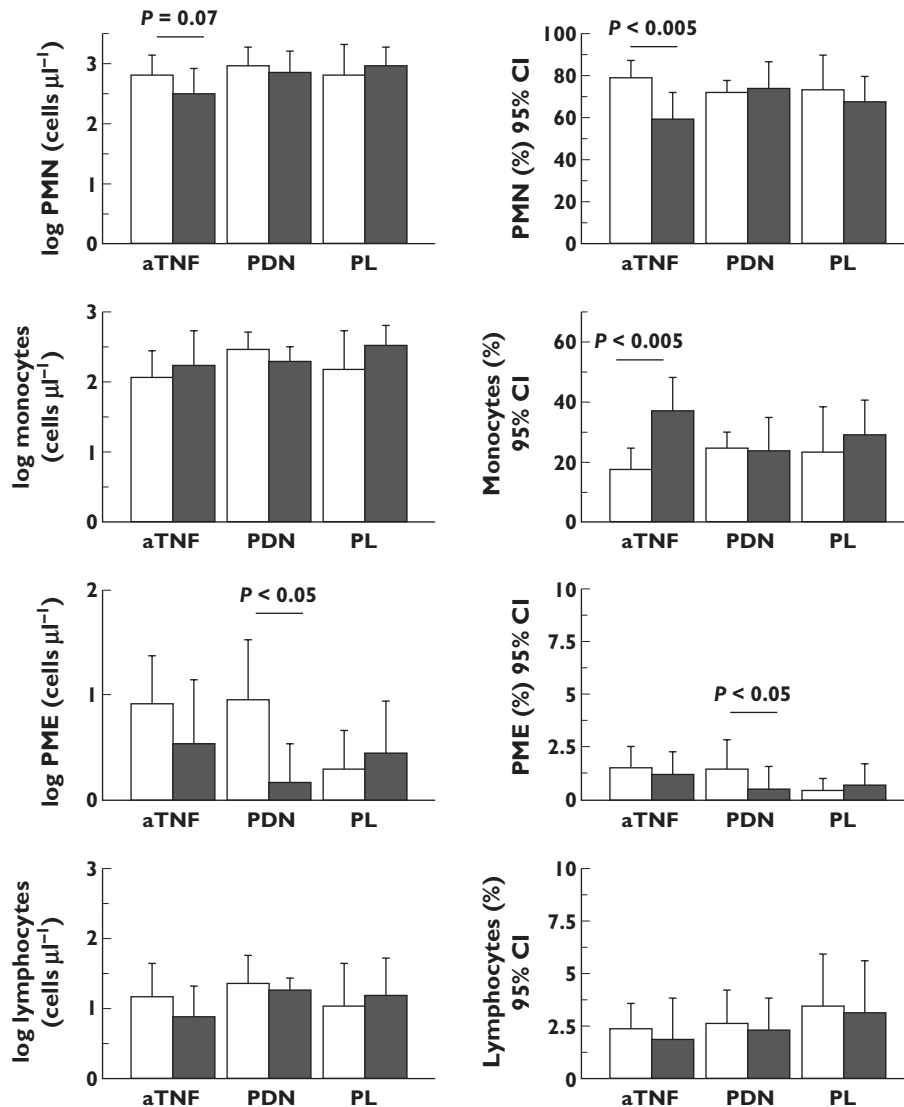


Figure 3

Cell counts before (white bar) and after treatment (black bar). Data are expressed as means +95% CI. Paired t-tests were used to assess whether mean changes from day 1 to day 7 were significantly different from zero by treatment. aTNF, anti-TNF; PDN, methylprednisolone; PL, placebo. PME, polymorphonuclear eosinophils; PMN, polymorphonuclear neutrophils

[11]. However, in the latter paper, the exudates were highly variable in the same subjects, probably because the method was not standardized, and the blisters were amplified by addition of bacterial antigens [11]. More recently, the cantharidin technique was revisited by Day *et al.* who reported a standardized procedure [3]. In a group of 12 healthy subjects they showed that the 24 h cantharidin-induced blister was 1–2 cm diameter, 100–800 μl volume, about 2500 cells μl^{-1} with a viability of 92–100% and a majority of PMN/PME (60–90%), those characteristics being consistent with our results. The same study reported the presence of several inflammatory mediators in the blister fluid (mainly IL-8, ENA-78, GRO- α , C3a, TNF, IL-12, IL-1b [3].

The safety and tolerability of the cantharidin-induced blister challenge is very good. Discomfort, without pain, occurs for 2 days over the area of blistering. While the kinetics of leucocyte trafficking is subject-dependent [12], in our experience (see Table 1) the blister diameter and the weight of the liquid obtained at 24 h is very homogeneous between subjects. In our experience, the skin heals within 5 weeks. Hyper-pigmentation can occur, but persists no more than 6 months [3]. In fact, the concentration of cantharidin used in this study equates 25 mg per blister, a seven-fold dilution of the pharmaceutical preparation used for plantar verrucae removal. There are very rare case reports of patients who developed acute cellulitis and/or lymphangitis soon after topical applica-

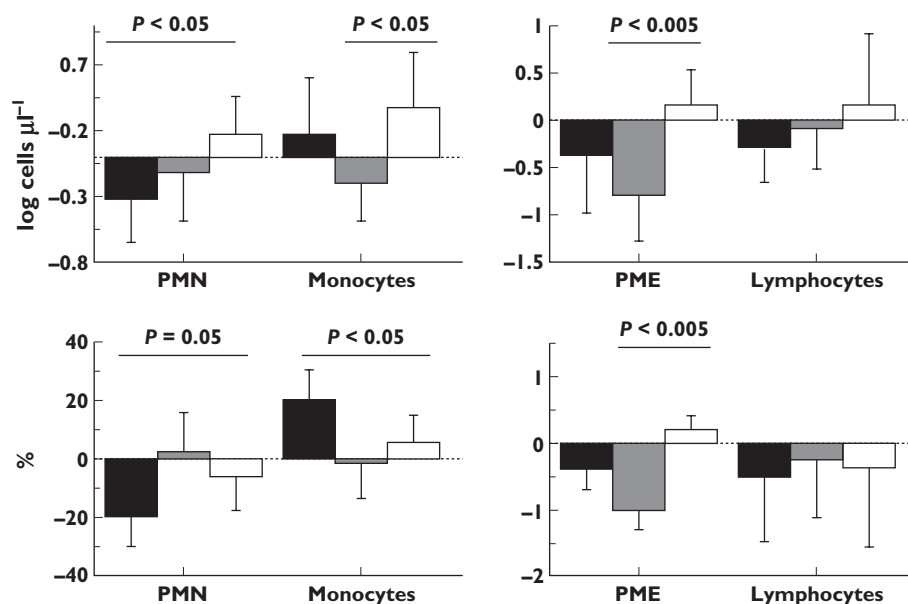


Figure 4

Comparison of the change of blister cell counts after each treatment. The black bar indicates the change after treating with anti-TNF, the grey bar is the change after methylprednisolone and the white bar is placebo. Data are expressed as means \pm 95% CI. ANOVA among the three treatments was applied followed by comparisons among the treatments. PME, polymorphonuclear eosinophils; PMN, polymorphonuclear neutrophils

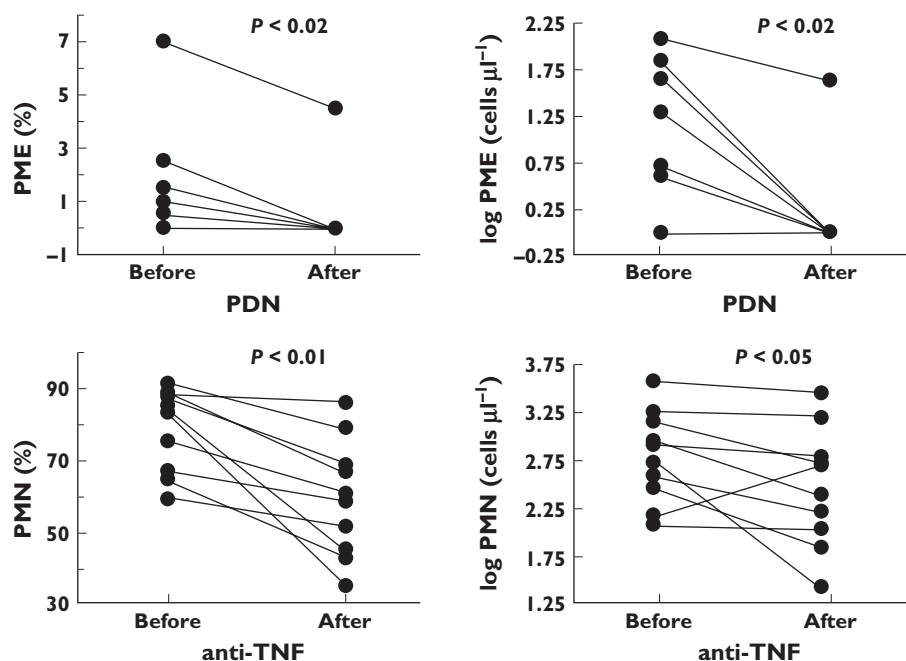


Figure 5

The drug effects in each subject. The absolute number (log transformed) and % of neutrophils (PMN) and eosinophils (PME) measured in the cantharidin-induced skin blister were compared by paired *t*-tests. PDN, methylprednisolone

tion of cantharidin for eradication of plantar verrucae [13]; therefore susceptible patients, in particular those with diabetes or peripheral arterial diseases, should not be studied.

Few data are available on the intra-subject reproducibility of the technique. Recently, Morris *et al.* have reported, in five subjects, that the total number of leucocytes is equivalent to two cantharidin-induced blisters at 1

week's interval [12]. Our results show that the intra-subject reproducibility at a 7 days' interval is significant for the total leucocyte count and their differential and for the size of the blisters. This observation is important to validate this technique as a human non-invasive model of innate immunity [2] which could assess the anti-inflammatory properties of novel drugs. To assess the anti-inflammatory potential of novel compounds in healthy subjects given the selected immunological challenges, it is important to know the effect of proven reference therapies currently part of the standard of care for inflammatory diseases.

In the current study, the tested anti-inflammatory agents included methylprednisolone and an anti-TNF agent. Methylprednisolone is a synthetic corticosteroid with a large spectrum of anti-inflammatory properties, reducing tissue eosinophils in allergic responses by an apoptotic effect and an inhibitory effect on the medullar production [14]. In contrast to the effects on eosinophils, methylprednisolone increases the number of tissue neutrophils by increased survival [14]. Consistently, in clinical practice, methylprednisolone is efficacious in eosinophilic inflammation, such as allergic asthma, whereas it fails to interfere with neutrophil-related inflammatory diseases such as chronic obstructive pulmonary disease. Therefore, the inhibiting effect of methylprednisolone at a daily dose of 20 mg for 7 days (which is within the recommended dose range for severe asthma) on eosinophil number, accompanied with a rise of the percentage of neutrophils in the liquid of the cantharidin-induced blisters, is not a surprise. Other eosinophilic models are available, such as the allergen-induced nasal/bronchial inflammation. However the skin blister model is a tissue window on the inflammatory mechanisms, while the allergen induced response explores the inflammation outside of the tissue (at the extra-mucosal level).

Adalimumab is a complete human IgG1 anti-TNF α mAb which binds to the soluble and transmembrane forms of TNF α with high affinity, thereby preventing TNF α from binding to its receptors. *In vitro* studies have shown its effect on the induction of cell lysis and apoptosis. Exposure to adalimumab restores normal chemotactic neutrophil activity, decreases the influx of leucocytes to the inflamed joints, and reduces the activation marker CD69 on neutrophils in patients [15]. Consistently, in the present study, at a dose of 40 mg (which is the recommended maintenance dose for severe rheumatoid arthritis, Crohn's disease and psoriasis), adalimumab attenuates the cantharidin-induced blister neutrophilic inflammation.

Since we did not observe any effect which was common to the two test drugs in the cantharidin-induced blister challenge (in particular the monocytes), this method is not suitable as a general test of anti-inflammatory activity. However, for drugs that have been shown by other methods to possess anti-inflammatory activity, the cantharidin blister challenge can be used to characterize the cellular response.

Recently, the cantharidin-induced blister has been used as tissue injury model to demonstrate that aspirin, at low dose prevents leucocytes from accumulating by triggering epi-lipoxin [4] and that two phenotypes exist with regard to the kinetics of the inflammatory response to cantharidin which are associated with the blocking effect of aspirin [12]. Those observations also suggest that the cantharidin-induced skin blister could be a promising model to evaluate anti-inflammatory agents in humans.

Another interesting feature of the skin blister is that it is a suitable technique for the assessment of peripheral compartment pharmacokinetics (PK), e.g. in saliva [16]. The PK profile of an antibiotic (meropenem) in blister fluid is also different compared with plasma. Therefore, the PK evaluated at the blister fluid level supports the dose in the management of skin and soft tissue infections [17].

The blister model could also contribute to understanding the mechanisms involved in some inflammatory diseases, such as Crohn's disease [18].

In conclusion, the cantharidin-induced blister is a safe and well tolerated, local inflammatory reaction, with a low inter-subject variability of the amplitude, a high intra-subject inter-day reproducibility and is inhibited by specific (anti-TNF) and non specific (corticosteroids) systemic anti-inflammatory agents. This model could be of interest to evaluate anti-inflammatory agents in their early phase development.

Competing Interests

There are no competing interests to declare.

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